

Detection of Anthelmintic Resistance to Gastrointestinal Nematodes in sheep: Laboratory and Survey Investigation in Khartoum State

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Dedication

To the spirits of my father, mother, to my teacher Dr/ Gundi and my brothers and sisters I dedicate this work.

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Abstract

The present study was conducted mainly for evaluation and tackling the problem of anthelmintic resistance and the appropriate tests for measuring it in the field, under Sudan condition. A survey of gastrointestinal nematodes of sheep was made and 796 faecal samples were collected in Khartoum, State and examined during the period June 2005 – May 2006.

In the present study sheep were found to be infected by different types of parasites eggs. These were Strongyles, *Strongyloides* spp, *Trichuris* spp, *Monezia* spp and *Coccidia*. The peak of nematodes eggs count occurred in October.

Identification of infective third stage larvae from faecal samples cultures revealed the following; *Heamonchus contortus*, *Oesophagostomum columbianum*, *Trichostrongylus* spp and *Strongyloides papillosus*.

In the *in-vitro* test of anthelmintic resistance, the ED₅₀ obtained from larval paralysis assay (LPA) was 0.890862 µg/mL for levamisole powder and 0.000107 ng/ml for abamectin injection. The results revealed

the resistance for levamisole and no resistance was detected in abamectin. In egg hatch assay (EHA), the ED₅₀ showed resistance of 482.444521 ng/ml for ivermectin and 256.525577 ng/ml for doramectin and 6.924595 µg/mL for levamisole. but albendazole 0.003162 µg/mL and abamectin 7.410285 ng/ml showed no resistant.

المستخلص

أجريت هذه الدراسة للتقييم و التعامل مع مشكلة مقاومة الأدوية طاردات الديدان الاسطوانية والاختبارات اللازمة لقياسها تحت ظروف السودان. تم عمل مسح داخل ولاية الخرطوم وجمعت 796 عينة من روث الضأن في الفترة من يونيو 2005 الى مايو 2006. وأظهرت نتائج الفحص إصابة الضأن بأنواع مختلفة من بيوض الطفيليات وهي بيوض الاسترونجايل، بيوض الاسترونجلوديس ، بيوض التراكيورس ، بيوض المونيزا و الأكياس البيضية للكوكسيديا.

تشخيص الطور المعدي اليرقي الثالث من المزارع البرازية أوجد الآتي :-

هيومنكس كونتورتس، اوسوفاقوستوموم كولمبيانوم، استرونجلويديس بابيلويسس ونوع ترايكوسترونجيلس. كذلك أوضحت الدراسة أن قمة الإصابة بالديدان الاسطوانية تكون في أثناء شهر أكتوبر .

في الاختبار غير الحي لكشف مقاومة طاردات الديدان أجرى اختبار الشلل اليرقي وجدت الجرعة المؤثرة للعدد 50 بالنسبة لعقار بدرة اليفاميزول 0.890862 ميكروجرام/مل و0.000107 نانوجرام /مل بالنسبة لعقار الابامكتين حقن . هذه الدراسة أظهرت عدم وجود مقاومة لعقار بدرة اليفاميزول وكذلك لعقار الابامكتين حقن. وفي اختبار الفقس البيضي أظهرت الجرعة المؤثرة للعدد 50 مقاومة الديدان عند استعمال عقار الايفرمكتين حقن 482.444521 نانوجرام/مل و الدورامكتين حقن 265.525577 نانوجرام / مل وعقار بدرة اليفاميزول 6.924595 ميكروجرام/مل. كذلك أظهرت الجرعة المؤثرة للعدد 50 عند استعمال عقار

الابامكتين 7.410258 نانوجرام / مل وعقار البندازول شراب 0.003162 ميكروجرام/مل
وهذا يدل على عدم وجود مقاومة في كلا العقارين.

CHAPTER ONE

Introduction and Review of the literature

1.1 Introduction

Gastrointestinal nematodosis is a major health problem in sheep. It severely affects animal production and inflicts serious economic losses. The major gastrointestinal nematodes include *Tristrongylus* spp, *Haemonchu scontortus* and *teladorsagia* spp. Anthelmintic resistance in some nematode species from livestock has become a serious problem in several industrial countries and increasing number of cases of anthelmintic resistance are also being reported from third world countries (Geerts,S and Dorny,P.1995). Under intensive management system, anthelmintic drugs are used routinely to control nematode infections due to their relative ease of application and result in rapid development of anthelmintic resistant (Martin *et al.*, 1984).

Development of resistance in parasites of sheep is throwing amajor challenge to worm control practices. Resistance to benzimidazole anthelmintic was first reported in Australia (Smeal *et al.*, 1968). The introduction of ivermectin the early 1980 brought are volution in the control of animal parasites was accepted as a potent anthelmintic (Cambell and Benz, 1984). Unfortunately, resistance has developed to ivermectin and first indication for ivermectin resistance was reported from South Africa (Carmichael *et al.*, 1987) against *Heamonchus controtus* isolated from sheep. Subsequently, there has been world wide incidence of ivermectin resistance in various species of nematodes

(Gopal *et al.*, 1999). This burgeoning problem of ivermectin resistance in sheep parasites has drawn the attention of veterinarians and farmers to opt for alternative drugs and control strategies.

To detect anthelmintic resistance, *in-vivo* and *in-vitro* methods can be used. The most widely used test to assess anthelmintic efficacy is the faecal egg count reduction test (FECRT) (Coles *et al.*, 1992). Although this standardized test is valuable in detecting anthelmintic resistance, *in-vitro* tests are cheaper to perform, and therefore more suitable for large surveys. The egg hatch test (EHT) and the larval development test (LDT) are the most widely employed *in vitro* methods for detection of anthelmintic resistance in ovine nematodes under field conditions (Mitchell *et al.*, 1991; Hung *et al.*, 1992; Praslicka *et al.*, 1994; Bartley *et al.*, 2001; Ancheta *et al.*, 2004). The result of EHT and LDT are usually interpreted using ED₅₀ values (the concentration of drug producing 50% inhibition of hatching in EHT) or LC₅₀ values (the concentration of drug required to prevent the development of 50% of the eggs into infective (L3) larvae in LDT).

1.2. The objectives of this study:

- i. To provide information on the prevalence of gastrointestinal nematodes in sheep in Khartoum state.
- ii. To study *in-vitro* testss (Larval paralysis assay and egg hatch assay) when used Avermectin drugs (Abamectin injection, ivermectin injection and doramectin injection) compared with Albendazol drench and levamisole powder.

1.3. Literature Review:

1.4. Gastrointestinal nematode in the Sudan:

Helminthic parasites are known to prevail in Sudan (Gagoad and Eisa, 1968; Eisa and Ibrahim, 1970; El Badawi *et al.*, 1978; Atta El Mannan, 1983) and it is difficult to find an animal free of internal parasites (Magzoub, 1989).

Effect of parasitic disease include mortality losses, condemnation of meat, weight loss, depreciation of animal products and reduced resistance to other diseases as well as high expenditure on drugs (Holmes, 1985; Handayani and Qatenby, 1988; Magzoub, 1989; Chunlai *et al.*, 1995). gastrointestinal tract parasites are major cause of reduced productivity in ruminants throughout the world (Holmes, 1987).

Eisa *et al.* (1979) reported helminthes in sheep in the Sudan during the period 1902 – 1975. They reported 20 genera of helminthes parasites 5 genera of termatodes estimated (*Fasciola gigantica*, *Schistosoma bovis*, *Parmphistomum* spp., *Dicrocoleium* spp. and *Cotylophoron cotylophorum*), 6 genera of cestodes (*Avitellina* spp., *Monezia expansa*, *M. benedeni*, *Stilesia hepatica*, *Cysticercus tenuicollis*, *hydatidcyst*, *Coenorus cerebralis* and *Coenurus serialis*) and 9 genera of nematodes (*Bunostomun* spp., *Chabertia ovina*, *Cooperia pectinata*, *Gygeria pachyscelis*, *Heamoncus contortus*, *Trichuris ovis*, *Oesophagostomum columbianum*, *Strongloides papellosus* and *Tristrongylus axia*). Atta Elmanan (1983) in central region of Sudan found eggs of four genera of parasites during faecal examination, these comprised *Trichostrongylid* spp., *M. expansa*, *S. papillosus* and *Trichuris* spp. Ghada (2000) reported

Skrijabinema ovis and *Trichuris glabubosa*. Gasmir (2004) reported *Impalaia tuberculata* (Table 1).

Table 1: Helminth parasites of sheep reported in the Sudan

Name of worms	Record	
	Year	Place
<i>Bunostomum spp.</i>	1965	Khartoum
<i>Chabertia ovina</i>	1965	Kosti
<i>Chabertia ovina</i>	1969	El Obeid
<i>Cooperia pectinata</i>	1970	El Obeid
<i>Gaigeria paschycelis</i>	1957	Malakal
<i>Haemonchus contortus</i>	1949	Khartoum, Kosti
<i>Haemonchus contortus</i>	1956	Kordofan
<i>Haemonchus contortus</i>	1959	Malakal
<i>Haemonchus contortus</i>	1963	Darfur, Kassala
<i>Oesophagostomum columbianum</i>	1955	Khartoum, Kosti
<i>Oesophagostomum columbianum</i>	1965	Kordofan
<i>Oesophagostomum columbianum</i>	1967	Darfur
<i>Strongyloides papillosus</i>	1957	Darfur
<i>Strongyloides papillosus</i>	1969	El Obeid
<i>Trichuris ovis</i>	1955	Malakal
<i>Trichuris ovis</i>	1958	Malakal
<i>Trichostrongylus axei</i>	1956	Khartoum
<i>Trichostrongylus axei</i>	1960	Malakal
<i>Skrjabinema ovis*</i>	2000	Khartoum
<i>Trichuris globuosa*</i>	2000	Khartoum
<i>Impalaia tuberculata**</i>	2004	Khartoum

Source: Modified from Eisa, *et al.* (1979)

* Ghada, H.A. (2000)

** Gasmir, G.S. (2004)

1.5. Anthelmintics:

Anthelmintics are chemicals (drugs) used to treat and control parasitic worm or helminthes that inhabit the gastrointestinal tract, and other tissues and organs of animals and birds. They are thus distinguished from ecto-parasitical compounds, sometimes loosely termed insecticides, which act against external parasites such as mite, tick, lice, and fleas.

Helminths have lifecycles of varying complexity, ranging from the direct cycle of most roundworms (nematodes) which involve only one host, to the indirect cycles of tapeworms (cestodes) and flukes (trematodes) which may utilize two or more hosts (Brander. *et al.*, 1994). Anthelmintics are important in the control of the parasitic nematodes of grazing animals. In 1999, sales of antiparasitic agents worldwide were valued at \$ 3.49 billion, of which 53% was spent on anthelmintics (Coles, 2001), indicating the significance of worms. However, the regular use of anthelmintics has resulted in the development of anthelmintic-resistant nematodes, a problem which is most serious in sheep and goats in the southern hemisphere (Waller *et al.*, 1995, 1996; Vanwyk *et al.*, 1999), although resistance is also increasing in worms of cattle and horse (Coles *et al.*, 1999; Familton *et al.*, 2001).

There are only three broad spectrum anthelmintic groups available for treatment of grazing animals for the control of nematodes. Group 1, the benzimidazoles (BZ), group 2, the imidazothiazoles (Levamisole, LEV) and hydropyrimidines (pyrantel / morantel), and group 3, the macrocyclic lactones (ivermectins and milbemycins, ML), have different mechanisms of action (Coles *et al.*, 2006).

1.5.1. Benzimidazoles group:

Since their appearance in the early 1960s, the benzimidazole have been subjected to continuous structural modifications to improve their safety and spectrum of activity, culminating in drugs which show efficacy not only against most nematodes but also cestodes and trematodes. Because of their poor solubility benzimidazole are generally dosed orally as suspension, pastes or boluses, or as powders, granules and pellets for mixing with the diet.

All benzimidazoles have a good activity against nematodes, their larvae and eggs; the more recent analogues have some tape worm activity (mebendazole is highly effective against larval tapeworms) and albendazole is also effective against adult liver fluke. However, the most recent derivative to appear, triclobendazole, is quite different. It is highly effective against all stages of liver fluke, from one day old to adult, but has no round worm activity.

With few exceptions, these drugs are very safe anthelmintics having a high therapeutic index, but care should be taken when dosing pregnant animals because the benzimidazoles have been shown to produce embryotoxicity and teratogenicity (Brander *et al.*, 1994).

1.5.1.1. Albendazole:

Albendazole, methyl [5-(propylthio)-1H-benzimidazole-2-yl] carba-mate, is widely used for treating ruminant roundworms and flukes.

1.5.1.1.1. Activity:

Albendazole is active against all important nematodes and their larvae, including hypobiotic or inhibited forms. It is also effective against tapeworms and adult fluke.

1.5.1.1.2. Metabolism:

The drug is rapidly metabolized to sulphone and sulphoxide which may prevent the liver fluke and tape worm activity. In sheep, 51% of the dose is excreted in the urine, mostly in the first 48 h. Very little albendazole remains unmetabolized. Drug residues persist for many days; consequently, a period of 10 days from dosing must elapse before sheep may be slaughtered (14 days for cattle) for meat, and cows producing milk for human consumption should not be treated.

1.5.2. Imidazothiazole group:

The water-soluble anthelmintic tetramisole provided the advantage over other drugs then available that it had excellent gastrointestinal roundworms and lungworm activity and could be administered by injection. It was soon realized that tetramisole was a mixture of two optically active isomers, of which the laevorotatory (L) isomer, levamisole was responsible for its efficacy against nematodes. The corresponding dextrarotatory (D) isomer (dexamisole) had little anthelmintic efficacy, and its safety profile was no better than levamisole. Butamisole, a derivative of m-aminotetramisole was used for a short period as a dog round worm remedy, but was withdrawn on account of its toxicity (Brander, *et al.*, 1994).

1.5.2.1. Levamisole:

Levamisole, 6-phenyl-2, 3, 5, 6-tetrahydroimidaza (2, L-b) thiazolehydrochloride, is a potent water soluble roundworm remedy which may be administered orally, parenterally, or percutaneously.

Levamisole is essentially a cholinergic agent and as such, causes paralysis of the worms. It has also been shown to inhibit the enzyme fumarate reductase but this is unlikely to be its mode of action *in- vivo*. The immunomodulatory properties of this drug have received much attention in the field of human medicine.

The safety index of levamisole is fairly low, but it is safe to use in pregnant animals, and its rapid absorption and excretion permits a short withdrawal period. The most popular route of administration to cattle is by injection, but the drug may also begin orally by drenching or be added to the drinking water. 'spot-on' formulations for topical application are quite widely used (Brander *et al.*, 1994).

1.5.2.1.1. Activity:

Levamisole is effective against adult and larval gastrointestinal roundworms and lungworms, both orally and parenterally. Success against canine heart worm microfilariae. It was a very rapid effect on parasites, expelling most worms within 24 h (Brander *et al.*, 1994). In sheep used against the following nematode infection-stomach worms (*Haemonchus* spp., *Trichostrongylus* spp, and *Ostertagia* spp.), intestinal worms (*Trichostrongylus* spp, *Coopeeria* spp., *Nematodirus* spp. *Bunostomum* spp., *Oesophagostomum* spp., *Chabertia* spp.) and lungworms (*Dictycaulus* spp.) (Parasiticides – livestock html).

1.5.2.1.2. Metabolism:

Levamisole is very rapidly absorbed whether given orally or by injection, but the parenteral route produces higher blood levels of drug. Peak blood levels occur within 1 h. of dosing, with a plasma half-life of no more than 4 h. Under alkaline conditions it hydrolyses to the insoluble metabolic OMPI (2-oxo-3 – (2-mercaptoethyl)-5-phenyl imidazo-lidine). The drug is rapidly eliminated from the body (46% in urine and 32% in faeces within 24 h) and consequently, has a short withdrawal period of 3 days for meat and 1 day for milk (Brander *et al.*, 1994).

1.5.3. Macrolides group:

The avermectin probably represents the biggest break through in parasitic control since the discovery of benzimidazoles in 1960s. Avermectin and closely related milbemycins, are antibiotics produced by actinomycete microorganisms, and are termed macrocyclic lactones (or macrolides) (Brander *et al.*, 1994). In addition to being highly potent, broad-spectrum nematocides, avermectins (AVMs) milbemycins are also potent insecticides and acaricides (Campbell *et al.*, 1983). The natural AVMs and milbemycins are produced by soil-dwelling *Streptomyces* spp (Burg *et al.*, 1979 and Takiguchi *et al.*, 1980).

Structurally the AVMs and milbemycins are loosely related classes of 16-membered macrocyclic lactones. The most important structural difference between them lies in the presence in the AVMs of a disaccharide substituent at C-13.

The range of antiparasitic activity of avermectins is staggering. at a single oral, subcutaneous or intramuscular dose of 0.2 mgkg⁻¹

avermectin is active against the adults and larvae of all major round worm parasites of sheep, cattle and horses, including inhibited larvae of *Oestertagia* in cattle and the tissues-dwelling larvae of horse strongyles.

Avermectins have no activity against platyhelminths (Flukes and tape worms), a phenomenon that could be readily explained from their reputed mode of action. These drugs have been shown to cause paralysis by affecting γ -aminobutyric acid (GABA) mediated signals between nerves and muscles. Flukes and tape worms are thought not to use GABA as a neurotransmitter (Brander *et al.*, 1994). Macrocyclic lactones including doramectin (DRM), ivermectin (IVM), moxidectin (MXB) (Barber *et al.*, 2003) and abamectin (ABM) (Dur-Zong Hsu, *et al.*, 2001) only MXD and IVM are registered for use in sheep via the oral administration however, results in a relatively low bio-availability of drug because of binding with organic matter in the sheep's rumen (Ali and Hennessy, 1996; Hennessy *et al.*, 2000). Which has also been demonstrated in cattle (Alvinerie *et al.*, 1993) Hence subcutaneous (S.C.) delivery of MLs in sheep offer an alternative to oral treatment, reducing the need for withholding feed pretreatment (Hennessy, 1997).

1.5.3.1. Abamectin:

Abamectin an analog of Ivermectin, is a mixture of a vermetins containing at least 80% avermectin B_{1a} and less than 20% avermectin B_{1b}, which derived from the soil bacterium *streptomyces avermeitilis* (Campbell *et al.*, 1983; Agarwal, 1998). Abamectin was widely employed to control insects and mites of a wide range of agricultural products such as fruit, vegetable and ornamental crops (Laukas and Gordon, 1989). Unlike IVM, ABM has a double bond at C₂₂ – C₂₃. These

highly lipophilic compounds have wide antiparasitic spectrum of activity (Fisher and Mrozik, 1992; Campbell, 1993).

1.5.3.1.1. Activity:

Abamectin is generally highly effective in controlling gastrointestinal and lung worm larvae, adults and hypobiotic stage, but ineffective against flukes and other flat worms (Marriner, 1986; Campbell, 1993). Abamectin also very effective in controlling some ectoparasites.

1.5.3.2. Ivermectin:

Ivermectin is a semi synthetic derivative of avermectins B, which is a fermentation product of the actinomycete, *Streptomyces avermectilis* (Millar *et al.*, 1979).

It was introduced to market place as anti-parasitic drug in 1981. Its worldwide acceptance in livestock production and in the health care of companion animals has made it a major commercial success. Its efficacy in human onchocerciasis (river blindness) has made it a promising candidate for the control of one of the most insidious and intractable of tropical disease (Campbell, 1989).

1.5.3.2.1. Activity:

Ivermectin is effective against all stages of every major parasitic nematode, including canine heart worm larvae. It is also a potent ectoparasiticide, but has no activity against tape worms or flukes (Brander *et al.*, 1994).

1.5.3.2.2. Metabolism:

Ivermectin is readily absorbed, especially when given parenterally. Peak plasma concentrations are reached 4.4 h after dosing directly into the abomasum (with 100% bioavailability), but 23.5 h after dosing intraruminally, which suggests that the drug is rapidly metabolized in the rumen-high concentrations of the drug and sustained in the tissues for long period, particularly after parenteral administration. Drug residues occur mainly in the liver and fat with very little in the muscle. The bulk of drug is excreted in the faeces 98% with only 2% in the urine.

A withdrawal period of 21 days (28 days for cattle) before slaughter is required because of the persisting levels of drug in tissues, and ivermectin must not be used in dairy cows providing milk for human consumption (Brander *et al.*, 1994).

1.5.3.3. Doramectin:

Doramectin is a new avermectin derived from the soil-dwelling actinomycetes. It was approved for use in beef cattle in United States (Schenck and Lagman, 1999). Doramectin and ivermectin have broad nematode and arthropod spectra of activity (McKellar and benchaoui, 1996).

1.5.4. Modes of anthelmintic action:

Benzimidazole anthelmintics have been shown to inhibit the enzyme system fumarate reductase, also to inhibit glucose uptake and cause depletion of the parasites glycogen reserves, but their true *in-vivo* activity is likely to rest with their ability to polymerize tubulin in the microtubules of cells, which affect the worms ability to digest and absorb

nutrients (Brander *et al.*, 1994). Yet another recent theory is that benzimidazoles act by increasing the permeability of cell membranes to protons (McCracken *et al.*, 1982).

The effects of ivermetin and moxidectin on pharyngeal pumping in *H. contortus* has shown that both drugs may share common action mechanism but that there may be subtle differences in the response to the target site between these compounds (Paient *et al.*, 1999). Macrocyclic lactone antiparasitics produce aflaccid paralysis of the somatic worm musculature and inhibit feeding of the parasite by locking pharyngeal pumping (Geary *et al.*, 1993; Martin *et al.*, 1996; Kotze, 1998; Sangster and Gill, 1999). The latter effect is exhibited of chemotherapeutically relevant levels, and it has therefore been suggested that disruption of ingestive activity and worm starvation is the real nematocidal action of these compounds (Sangster and Gill, 1999; Paient *et al.*, 1999).

Most of the commercially available antinematodal drugs exert their effect on the nervous system of the parasite. Members of one of this drug category act as acetylcholine agonists and include levamisole, the tetrahydropyrimidines and some other structurally related compounds. Recent studies using electrophysiological techniques have shown that the surface of somatic muscle cells of nematodes possess nicotinic acetylcholine receptors (nAChR) that can be opened by the nicotinic anthelmintics (Evans, and Martin, 1996; Martin *et al.*, 1996, 1998). Binding of these compounds to the recognition site of the excitatory receptor produces depolarization and spastic paralysis of the nematode

muscle that can result in parasite expulsion. The nAChR of vertebrates is a thoroughly investigated receptor operated cation channel, composed of a pentameric structure built up of a combination of different subunits (Unwin, 1995). Each α Chain of the channel contains a binding site for acetylcholine. The subunit composition and stoichiometry of this receptor can vary between different subtypes resulting in a functional diversity of nAChRs. Details of the biochemical nature of the nematode nAChR have not yet been revealed, but the subunit sequence features and pharmacological profile of this channel resembles vertebrate nAChRs (Martin *et al.*, 1997).

1.6. Anthelmintics resistance in the world:

Anthelmintics resistance is an increasing problem for the sheep industry in New Zealand, since the first reported case in 1980 (Vlasseff and Kettle, 1980), resistance has become relatively common place; approximately 60% of sheep farms have detectable levels of resistance to one or more anthelmintic families (McKenna *et al.*, 1995). Until recently, reports of resistance to the macrocyclic lactone family of anthelmintics have been largely restricted to parasite of goats (Badger and McKenna, 1990; Gopal *et al.*, 1999; Leathwick, 1995; Pomroy *et al.*, 1992; Watson and Hosking, 1990). To a lesser extent, cattle (Vermunt *et al.*, 1995).

Although reliable data are scarce, it is clear that resistance to all of the broad-spectrum anthelmintic families is more prevalent in goats than in sheep (Kettle *et al.*, 1983; McKenna 1991), a phenomenon which may be attributable to more rapid metabolism of the drugs in the former species (Hennessay *et al.*, 1993, Kettle and White, 1982; McKenna, 1991)

and/or to the highest frequency of treatments applied to goats compared with sheep (Mason, 1997).

The first cause from the south Island, was reported by (Mason *et al.*, 1999) and the second was reported by Leathwick *et al.* (2000). In the UK, the full extent of anthelmintic-resistant nematodes in sheep flocks is not known. In Scotland, 81% of low land and 45% of hill farms has benzimidazole resistant nematodes but not other types of resistance (Bartley *et al.*, 2001). Already in some goat herds there are nematodes resistant to all three anthelmintic groups (Coles *et al.*, 1996) and a similar situation has recently been reported on Scottish sheep farm (Sargison *et al.*, 2001). If animal health and productivity are to be maintained, it is clear that anthelmintic efficacy must be preserved because no equally effective methods of nematode control are available. Recommendations have been made for preserving anthelmintic efficacy (Coles and Roush, 1992; Herd and Coles, 1994) but, at least by sheep farmers, they are not being widely followed (Coles, 1997). The problem with some of the recommendations, for example, the benefit of alternating the use of different anthelmintic groups annually, is that they have never been validated experimentally. The lack of interest in antiparasitic drug resistance from almost all the bodies within the UK that provide support from veterinary research is the major reason for the shortage of information on both epidemiology and the optimal management of anthelmintic-resistant nematodes. At the same time as resistance is developing to anthelmintics, the liver fluke, *Fasciola hepatica* is developing resistance to fasciolicides, and particularly to tricla-

bendazole, the most effective drug on the market (Mitchell *et al.*, 1998; Thomas *et al.*, 2000).

There is no question of the seriousness of the problem in Australia, South Africa and the humid semi-tropical regions of South America where about 300 million sheep are raised, and the scientific literature is well served with detailed reviews of the prevalence, the rate of spread and the increase in magnitude of the resistance (Martin *et al.*, 1998; Waller, 1986, 1987; Prichard, 1990; Boray *et al.*, 1990) these reviews explain why anthelmintic resistance is apparently so much greater in the southern Hemisphere; the problem is clearly linked to the frequency of anthelmintic treatment, to the relative importance of the nematode species (being of greatest importance in the regions endemically infected with *Haemonchus contortus*) and the prevailing type of grazing management (set-stocked on permanent pasture). In Australia the last 25 years have seen resistance emerge as the most important disease problem confronting the sheep industry in Australia (Anon, 1989). Resistance was first reported in 1968 in *Haemonchus contortus* to thiabendazole on three sheep farm in North New South Wales by Smeal *et al.* (1968). The prevalence of benzimidazole resistance rapidly increased in the summer rainfall regions with outbreaks of haemonchosis due to drug-resistant worms being reported in the mid 1970.

The most recent study on German horses (Beelitz and Gothe, 1997) confirmed earlier reports of benzimidazole resistance (Bürger and Baver, 1987, Ullrich *et al.*, 1988), but found that the tetrahydropyrimidine pyrantel and the macrocyclic lactone ivermectin

were fully effective; in general, there appears to be less resistance to pyrantel than to benzimidazole (Tarigo-Martinic *et al.*, 2001).

The development of strongylid nematode resistance to modern anthelmintics has been observed for over 25 years, resistance to ivermectin, a novel chemical class, has developed only recently. The first indication of the development of ivermectin resistance by *Haemonchus contortus* in sheep occurred in 1986 in South Africa. Carmichael *et al.* (1987) and Van Wyk and Malan (1988) isolated seven apparently distinct strains of *Haemonchus contortus* to ivermectin that had developed resistance under normal farming conditions in various regions of South Africa. Van Wyk *et al.* (1989) presented additional data on four previously reported isolates and the probable existence of five additional ivermectin resistant strain of *Haemonchus contortus*.

Eschevarria and Trindada (1989) isolated an ivermectin resistant strain from sheep raised on pastoral experimental station in southern Brazil. Craig and Miller (1990) isolated an ivermectin resistant strain from an Angora goat flock in southern Texas. In Spain, the first anthelmintic resistance recorded occurred after Cashmere goats were imported from United Kingdom, Requaio *et al.* (1997), where high prevalence of anthelmintic resistance in fibre producing goats had been recorded, Jackson *et al.* (1992). The same situation was observed in Angora goats imported to Slovakia from New Zealand, Varady *et al.* (1993) and sheep imported to Greece from Great Britain, Himonas *et al.* (1994). In French dairy goat farms, no infected animals are introduced after the flock has been set up. therefore expected that dairy goat farms

are isolated as far as gastrointestinal nematodes are concerned. It has been demonstrated that, in such isolated arms, the constitution of the flock is a critical step for the introduction of anthelmintic resistant parasites, Silvestre (2000). In Morocco, Ait-Baba *et al.* (1999) reported that 60.5% of the alimentary needs of small ruminants are obtained from collective natural pastures, which is source of unknown and possibly resistant nematodes.

In Sudan, Osman *et al.* (1990) have designed the experiment in nyala to put forward strategic control for gastrointestinal nematodes outbreak in the area. Regular faecal egg counts were made during the dry and wet season. Gasmir (2004) found that faecal egg count reduction test of anthelmintic treated groups showed 100% efficacy for ivermectin and levamisole, while the Albendazole showed efficacy of 97% which is considered low resistance.

Table (2) and (3) explain the first reports of anthelmintic resistance in nematodes of sheep drug with different modes of actions, and reported cases of resistance in anthelmintic parasites of sheep and goat according to class of anthelmintic respectively.

1.6.1. Development of resistance:

In entomology it is widely accepted that for crop protection areas or fields are left untreated with insecticide, so that there is a reservoir of unselected insects to produce the next generation coming solely from insects which have survived treatment. The principle of allowing organisms to escape selection for resistance by a chemical is thus not a new idea, although it has not been applied significantly in helminth

control. The effectiveness of this tactic is increased considerably if the susceptible insects have a superior biological fitness over resistant insects, whether this difference occurs with parasitic helminthes is not known, but it does not appear to apply to benzimidazole resistance in *Teladorsagia (Oestertagia) circumcincta* (Barrett *et al.*, 1998; Elard *et al.*, 1998). Kelly *et al.* (1978) suggested that benzimidazole resistant *Haemonchus contortus* was fitter than the susceptible isolate with which it was compared, although with only two isolates, the difference may have been unrelated to drug resistance. Insects, of course, can fly from area to area, whereas nematodes are largely moved with animals, and thus untreated population of worms would have to be maintained on individual farms.

The most important factor in the development of resistance to an anthelmintic is the contribution that the worms which survive treatment become resistant with its second generation. This in turn depends on the numbers of worms in refugia, that is, the numbers of worms that are not exposed to anthelmintic. Vanwyk (2001) argued strongly that it is this factor, rather than the frequency of treatment and the lack of compliance with past recommendations, that is crucial in the development of resistance. He observed that refugia have been ignored by many publications on anthelmintic resistance. Even this idea is not new, Martin *et al.* (1981) showed that refugia delayed the development of resistance and Michel (1985) warned that pressure on worms to develop resistance is strongly influenced by the relationship between anthelmintic use and grazing management. If the anthelminthic being used reliably gave a

100% cure rate in treated animals, resistance would not become a problem. The current recommendations suggest that highly effective anthelmintic gives an efficacy of more than 98% (Wood *et al.*, 1995), but this level of control may well be selected more quickly for resistance than an anthelmintic which is insufficiently active (< 80%).

There are three main factors that influence the number of worms in refugia. The first numbers of larvae on pasture in the past 'the dose and move' strategy, whereby animals are treated and moved to rested pasture where there are few infective larvae, has frequently been recommended as ineffective method of nematode control (Michel, 1969; Boag and Thomas, 1973). There is no doubt that this method is effective in reducing the loss of productivity due to larval challenge, and it has been used successfully, particularly with a move to fields from which silage or hay has been harvested. However, as the only contamination to reach the new clean field will come from worms which have survived treatment, this method of control is almost designed to select for resistance. On some Greek islands with hot dry summers, only one to two doses of benzimidazole each year were sufficient to select for resistance (Papadopoulos *et al.*, 2001).

In Australia, the treatment of animals during a drought was recommended as a very effective methods of nematode control, but its use could explain why resistance is worse there and in certain other countries in the southern hemisphere than in the UK. Observations in Western Australia, in the desert areas used for sheep production, have shown that resistance has developed to the macrocyclic lactones despite only one or

two doses having been used per year for up to four years (Besier, 1997). The idea that keeping pasture contaminated with nematode larvae is a good practice goes against what veterinary surgeons and farmers have been taught. It is never the less, essential if anthelmintics are to be kept effective.

One of the important recommendation for the control of the resistance is to avoid the introduction of resistant nematodes, and this could still be the major reason for the spread of anthelmintics resistant nematodes in the UK. However, the introduction of susceptible nematodes will be helpful because they will tend to dilute out any resistance already present. Papadopoulos *et al.* (2001) considered that the mixing of flocks on the Greek main land may help to explain the lack of resistance under these management condition. The introduction of susceptible *Haemonchus contortus* helped to reverse the development of anthelmintic resistance under certain conditions in South Africa (Vanwyk and Van Schalwyk, 1990). With careful application in the UK, this approach may be capable of restoring anthelmintic efficacy on sheep farms where not anthelmintic is effective or where there is resistance to two anthelmintic groups.

In the South Africa, with hot dry conditions killing the free-living larvae, this strategy may be easier to apply than in the UK, where the most conditions allow infective stages to survive for prolonged periods on pasture. Under these conditions, treated animals would have to be infected and moved to clean pasture, rather than returning to currently grazed pasture. However, before it can be adopted in the UK it must be

carefully evaluated in the field. The finding that benzimidazole resistant isolates do not revert to susceptibility despite the use of alternative anthelmintics for 15 years (Jackson *et al.*, 1998) does not invalidate this argument. Although it requires investigation, it is probable that, by the time benzimidazole resistance is recognized by a farmer, in part owing to the poor sensitivity of current detection methods, there would be few nematodes of the problem species with genes for susceptibility left on the farm making reversion impossible despite the use of another anthelmintic type. On eight farms in France with benzimidazole resistant *T. circumcincta*, three had no homozygous genes for susceptibility (Elard *et al.*, 1999). The potential benefit of introducing susceptible nematodes points to the need to know the disease status of any animals entering a farm or to practice quarantine while their disease status is established.

The second main factor influencing the number of worms in refugia is the percentage of animals treated. Only treating some animals on farm with anthelmintics has been proved to be very successful in delaying the development of resistance. On dairy farms it has been traditional to treat only first-year animals. The bulk of the nematode eggs reaching the pasture will have come from untreated second-year and adult animals and any nematodes surviving treatment in the first-year animals will make a negligible contribution to the overall contamination on the farm. This could be the major reason why anthelmintic resistance is uncommon in bovine nematodes (Coles, 2002) and explain why the use of boluses in the first-year animals has not produced widespread resistance, it follows that the worst from of management is to treat

second-year and adult cattle unless there is serious disease problem caused by nematodes. It also suggests that keeping the same pasture each year for first-year animals and not allowing older animals to graze this pasture will invite the selection of anthelmintic resistant nematodes. The finding of two cases of resistance to macrocyclic lactones in cattle nematodes in the UK (Stafford and Cole, 1999; Coles *et al.*, 2001) and its apparent high prevalence in New Zealand (Familton *et al.*, 2001) indicates that with incorrect management, resistance may become a practical problem. The much greater prevalence of resistant nematodes in sheep than in cattle can be explained, at least in part, by the treatment of adult sheep as well as lambs.

The third main factor influencing the number of worms in refugia is the killing of all developmental stages in the host. Inhibition in the gastrointestinal mucosa is an important way in which nematodes survive the winter or summer drought. If these stages are not effectively treated with anthelmintic, the young worms are in refugia and this should delay the development of anthelmintic resistance. In a population of sheep nematodes that has never experienced benzimidazoles, the percentage of worms with genes for resistance is not known. The history of susceptible isolates examined by Elard *et al.* (1999) for gene frequency was not stated. The level of resistance in some populations before they are treated with drugs may account for the rapid development of benzimidazole resistance in the nematode of sheep and horse. If genes for pyrantel and ivermectin resistance are naturally rare in the nematodes of horses, resistance should be slow to develop, because these drugs do not kill

inhibited *cyathostomes*. This is what has happened in practice. Because moxidectin is quite effective against inhibited larvae (Xiao *et al.* 1994; Bairden *et al.*, 2001), there will be fewer larvae in refugia and resistance to the macrocyclic lactones may be more likely to develop. Research in sheep suggests that moxidectin may select more quickly for resistance in *Haemonchus contortus* than ivermectin (Lejambre *et al.*, 1999). In contrast, the three major groups of anthelmintics, benzimidazoles, levamisole/pyrantel and macrocyclic lactones are effective against inhibited larvae in sheep which may partly explain why resistance is so common. In cattle, levamisole is not usually very effective against inhibited larvae and in New Zealand resistance in bovine nematodes has apparently been confined to benzimidazoles and macrocyclic lactones (McKenna, 1996).

1.6.2. Other factors selecting for resistance:

The frequent use of anthelmintics will increase the rate of selection for resistance (Martin *et al.*, 1984), because the worms that survive treatment will have a greater chance than susceptible worms of contributing to the next generation. The rate at which resistance develops will depend on the level of pasture contamination during the treatments. Many worms on the pasture mean that more are in refugia. If the genes for resistance are very rare, reducing the worm burdens and pasture contamination to very low levels could mean that few worms are available for selection, which might reduce the risk of resistance developing. The genetics of resistance will also be important. Resistance will develop more quickly if the gene for resistance is dominant.

Ivermectin resistance dominant in both *Haemonchus contortus* (Le Jambre *et al.*, 2000) and *T.circumcincta* (Coles, 1996). There is very little information on the biology of resistant worms compared with susceptible ones. In *T.circumcincta* the benzimidazole-resistant worms appear to be as fit as the susceptible worms (Barrett *et al.*, 1998; Elard *et al.*, 1998), which is hardly surprising given the single mutational change in β -tubulin. If the resistant worms were less immunogenic and persisted longer than the susceptible worms, or if they produced more egg per worm per day than the susceptible worms, then they would have a reproductive advantage. This possibility has not been adequately investigated, but there is a suggestion that *Cooperia oncophora* that are resistant to macrocyclic lactones more pathogenic than susceptible worms (Coles *et al.*, 2001).

Table (2) Reported cases (r) of resistance in anthelmintic parasites of sheep and goat according to class of anthelmintic

Parasite	Bz	IMZ	ML	SAL	OP	TH
<i>H. contortus</i>	X	Rare	X	x	x	X
<i>Ostertiga spp</i>	X	x	X	-	-	-
<i>Trichostrongylies spp.</i>	X	x	X	-	-	-
<i>Nematodorus spp.</i>	x	-	-	-	-	-
<i>Fasciola hepatica</i>	x	-	-	x-	-	

Bz= Benzimidazole,

ML= Marocyclic lactone

OP= Organophosphate

IMZ= Imidazothiazole

SAL= Salicytarilid

TH= Thiophiophorate

Table (3) The first reports of anthelmintic resistance in nematodes of sheep drug with different modes of actions (Coles, *et al.*, 1994)

Year	Country	Drug	Nematodes
1957	USA	Phenothiazine	<i>H. contortus</i>
1964	USA	Thiabendazole	<i>H. contortus</i>
1968	USA	OP. compounds	<i>T.circumcinctus</i>
1976	Australia	Levamisole/morantel	<i>H. contortus</i>
1980	S. Africa	Raforaonide	<i>H. contortus</i>
1987	S. Africa	Ivermectin	<i>H. contortus</i>

OP= Organophosphate

1.6.3. Mechanisms of resistance:

1.6.3.1. Benzimidazole resistance in trichostrongyloid parasite:

Microtubules are major structural components of eukaryotic cells and are assembled from protomers composed of one α -tubulin polypeptide (Lacey and Prichard, 1986). In most metazoans, β -tubulin gene families consist of several single copy genes dispersed throughout the genome. β -tubulin sequences are well conserved in all eukaryotic cells with the greatest variation residing at the carboxy-termini (Litte and Seehaus, 1988). Despite close sequence homology, nematode and mammalian β -tubulins are different in their response to tubulin inhibitors: nematode β -tubulins bind to the benzimidazole more strongly than to mammalian tubulin. Benzimidazole binding to nematode tubulin alters the tubulin-microtubule equilibrium and causes depolymerisation of microtubules (Lacey, 1988). It further appears that a loss of high affinity tubulin binding for benzimidazoles in a population of nematodes is the mechanism of resistance.

Roos (1990); Ross *et al.* (1990) indicated that there was specific difference between benzimidazole-susceptible and benzimidazole-resistance populations of *H. contortus* when the genomic DNA of several of these population was analyzed.

Kwa *et al.* (1993) used probes to study the involvement of two separate β -tubulin loci in benzimidazole resistance in *H. contortus*. These authors found that the early stage of selection resulted in a decrease in the number of alleles of isotype 1 and that subsequent selection resulted in the loss of a second α -tubulin isotype 2, from the parasite genome.

The sequence of development of benzimidazole resistance in both *H. Contortus* and *T. colubriformis* proceed by first selecting an allele of isotype 1 which contains a mutation at residue 200-subsequent selection results in a deletion or at least changes in the region of primer compatibility in the gene encoding isotype 2.

1.6.3.2. Levamisole resistance:

In contrast to benzimidazole resistance, resistance to levamisole is inherited differently in *H. contortus* and *T. colubriformis*. In *T. colubriformis*, levamisole resistance is inherited as a sex-linked recessive trait (Martin and McKenzie, 1990). The sex determining mechanism in these nematodes is xx in females and xo in males (Lejambre, 1985), which means that a sex-linked recessive is recessive in the females but effectively dominant in the males as they have only one copy of the x chromosome. The inheritance of levamisole resistance in *H. contortus* was examined using mating and *in vitro* assay (Dobson *et al.*, 1996; Sangster, 1996) and in both studies it was concluded that levamisole resistance in *H. contortus* is inherited as an autosomal recessive and that more than one gene is involved.

Levamisole resistance appears to involve a loss of cholinergic receptors. In the free – living nematode *Caenorhabditis elegans*, several genes can confer levamisole resistance with mutations at any of the seven *Loic* , being able to confer extreme resistance (Lewis *et al .*, 1980).

1.6.3.3. Macrocyclic lactone resistance:

Macrocyclic lactone resistance includes resistance to the individual drugs in the class including ivermectin. Because of its historic use, the term ivermectin resistance is often used to describe macrocyclic lactone-resistant worm population. Macrocyclic lactone in field-selected strains of *H. contortus* is a dominant, autosomal trait, largely controlled by a single major gene (Lejambre *et al.*, 2000). Resistance to macrocyclic lactone ivermectin appears as side resistance to second generation macrocyclic lactones such as moxidectin (Barnes *et al.*, 2001). Macrocyclic lactone resistance in Australian macrocyclic lactone-resistant isolates manifests itself its ability to establish in sheep following treatment with moxidectin and ivermectin-sustained release capsules and this ability is dominant (Barnes *et al.*, 2001).

The major effect gene for macrocyclic lactone resistance in *H. contortus* has not been identified. Studies on *C. elegans* indicate that the macrocyclic lactone act on glutamate-gated chloride channels, binding to the α subunit (Arena *et al.*, 1995; Cully *et al.*, 1996). Blackhall *et al.* (1998) found a correlation between changes in allele frequencies of a putative α -subunit gene of *H. contortus* and resistance to macrocyclic lactones. One study of ivermectin binding in *H. contortus* identified a single high affinity binding site in membrane preparations but found no difference in binding to that site associated with macrocyclic lactone resistance (Rohrer *et al.*, 1994). This result does not discount the possibility that resistance to the macrocyclic lactone is related to a change at or near the site of action. Firstly binding in membrane

preparation may not necessarily be characteristic of binding to an in situ receptor (Sangster, 1996). Second, if the ion channel in question undergoes ligand-induced conformation change, alterations to areas of the α - or other subunits, unrelated to the macrocyclic lactone-binding site but blocking this conformational change may also confer resistance. A second lower affinity-binding site for ivermectin identified in similar membrane preparations from *H. contortus* (Gill and Lacey, 1998).

1.7. Methods for detecting anthelmintic resistance:

The extensive use of anthelmintic for the control of helminth infections on grazing livestock has resulted in the development of resistance that has become a major practical problem in many countries. Resistance in the field is usually suspected when there is an apparent poor clinical response to anthelmintic treatment (Kelly and Hall, 1979a, b). There are several factors to be taken into account before deciding on a diagnosis of anthelmintic resistance. First remember that a variety of conditions may cause clinical signs similar to those normally associated with parasitism. Secondly, anthelmintic may fail to control nematodes for a number of reasons other than resistance (Prichard and Hennessy, 1979; Prichard et al, 1980; Charleston, 1981) Failure in these cases can often be attributed to factors such as faulty drenching equipment or under dosing due to inaccurate assessment of body weight. The growing importance of anthelmintic resistance has lead to increased need for reliable and standardized detection methods (Coles *et al.*, 1992), some of which have been previously described and reviewed (President, 1985_b; Johansen, 1989; Taylor, 1991; Hazelby *et al.*, 1994).

Most of the methods described have draw backs either in terms of cost, applicability and interpretation of findings (Varady and Corba, 1999; Taylor, Hunt and Goodyear, 2002). The *in vivo* methods are suitable for all types of anthelmintics including those that undergo metabolism in the host to chemically active compounds (Taylor and Hunt, 1989). *In vitro* techniques offer rapid, sensitive and considerably more economic methods of screening but suffer from certain limitations (Taylor and Hunt, 1989).

1.7.1. *In- vitro* methods:

17.1.1. Egg hatch assay (EHAs):

Benzimidazole anthelmintics prevent embryonation and hatching of nematode eggs. A number of egg hatch/embryonation assay have been developed for the detection of resistance to this group of anthelmintics (Lejambre, 1976; Coles and Simpkin, 1977, Hall *et al.*, 1978; Whitlock *et al.*, 1980). As with the FECRT, guidelines for the use of EHA have been produced (Coles *et al.*, 1992).

Qualitative and quantitative interpretation of result can be made and identification of first or third stage larvae allows the species of nematode involved to be determined (Whitlock *et al.*, 1980). The essential aim is to incubate undeveloped eggs in serial concentration of the anthelmintic, usually thiabendazole it is the most soluble. The percentage of eggs that hatch (or conversely die) at each concentration is determined, corrected for natural mortality from control bottles, and a dose-response line plotted against drug concentration.

The requirement for under develop eggs in *in vitro* EHAs (Coles and Simpkin, 1977), has been a major obstacle to the application of the EHAs in routine diagnosis. As development proceeds beyond the ventral indentation stage, a false positive result may be obtained because sensitivity to thiabendazole decreases as embryonation proceeds (Lejambre, 1976; Weston *et al.*, 1984).

A number of techniques have been described to avoid the problems of screening for benzimidazole resistance in the field. Whitlock *et al.* (1980) described a method for assaying samples for benzimidazole resistance in the field. Worm eggs are recovered, on farm, by flotation in saturated sugar solution in McCartney flasks laid flat. After carefully decanting the sugar solution, the recovered eggs, adhering to the upper surface of the flasks, are then exposed to concentration of thiabendazole. During transport in the field, the flasks are carried in insulated boxes until controlled incubation facilities are available. Another method that aims to prevent the development of nematode eggs in transit to the laboratory, is to store faecal samples at 4°C for up to 3 days (Smith-Buijs and Borgsteed, 1986). Comparable results have been obtained by storing faecal samples in sealed polythene bags with the air excluded (Presidente, 1985_b). An anaerobic system has been described and used in UK which allows for the submission and testing of samples up to 7 days from the date of collection (Hunt and Taylor, 1989).

A modification of the EHA, the egg development test, has been described for determining the presence of benzimidazole resistance in *Nematodirus spathiger* (Obendorf *et al.*, 1986). Such a test could

conceivably be used for other nematodirus species.

An *in-vitro* EHA has been described by Dobson *et al.* (1986) for detecting resistance of nematodes to levamisole. Although more complex to perform, the assay yields data similar to those from EHAs for benzimidazole resistance.

1.7.2. Larval paralysis or motility assay :- (LPA)

In-vitro studies on motility can be used to determine the presence of resistance to anthelmintic with paralysing mode of action, such as levamisole, morantel and ivermectin. The test can be used to determine the LP_{50} . It can also be used to measure the effect of anthelmintic using special micro motility meter (Martin and Lejamber, 1979; Folz, Pax, Thomas, Bennett, Lee and Condex 1987). Boersema (1983) discussed the failure of this method to obtain repeatable results and suggested the reversibility of paralysis as a possible cause, Geerts, Brandt, Borgsteeds and Van Loon (1989), however, reported fairly good reproducibility of the test, any differences in repeatability being attributed to the age of larvae.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Survey:

Initial survey of gastrointestinal nematodes in sheep for identification of species involved. This was done by visits to the slaughterhouses and different places in Khartoum State.

2.2. Samples:

A number of 796 faecal samples were collected. The faecal samples were collected from different places in Khartoum State, from Omdurman Locality, Elsabloga Slaughterhouse, Omdurman Veterinary Hospital and different animal commercials. from Khartoum Locality, Gabal Awlia Slaughterhouse and Animal from the Central Veterinary Research Laboratories in Soba, from Khartoum North, Elkadro Slaughterhouse and Helat Koko. The faecal samples were examined for gastrointestinal parasites by using the modified Mc Master technique (MAFF) during the period June 2005 – May 2006.

2.3. McMaster counting chamber:

Without doubt this is the piece of equipment most frequently used in methods for estimating nematode egg counts in faeces. If difficulty is encountered with the purchase of this item it should be possible to manufacture it locally. It is possible to estimate the number of eggs using different multiplication factors with any one method depending on the dilution of the faeces and the exact area examined. Since both the ruled

grids and each chamber are precise measurements one can count the eggs under one grid, both grid, one chamber or the total area (both chambers). The volume under one grid is 0.15 ml a multiplication factor of either 15 or 30 is used depending on the number of grids examined. In use, the suspension of faeces to be examined is run into each chamber using a Pasteur pipette until it is full and then the chosen area is examined and all eggs seen are counted (MAFF, 1986).

2.4. Modified Mc Master method :

The use of a centrifuge facilitates examination by removal of fine particles and colouring. If a centrifuge is available this is the best method for routine faecal egg counts. Deposition of helminth egg is generally achieved by centrifugation at 1500 r.p.m. for 2 minutes although 1000 r.p.m. for 2 minutes will suffice (MAFF, 1986).

Method:

- i. About 45 glass balls are put in shaker jar and 42 ml water is added.
- ii. Three gram of faeces are put in the jar.
- iii. The jar is shaken until all the faecal matter is broken down.
- iv. The mixture is poured through a wire mesh screen with an aperture of 0.15 mm and the strained fluid caught in a bowl. The debris left on the screen is discarded.
- v. The strained fluid is stirred and the sample is poured into the centrifuge tube to within 10 mm of the top. The tube is centrifuged for 2 minutes at 1500 r.p.m. and the supernatant is poured off and discarded.

- vi. The tube is agitated until the sediment is loosened and forms a homogeneous sludge at the bottom of the tube. The tube is filled with saturated NaCl to the same level before.
- vii. The contents of the tube are thoroughly mixed by inverting it five or six times with the thumb over the end and a sufficient volume of the fluid is immediately withdrawn with a Pasteur pipette and carefully allowed to run into one chamber of the Mc master slide. After further mixing a second sample is withdrawn and run into the other chamber.
- viii. All the eggs under the two separate grids are counted. The number of eggs per g of faeces is obtained by multiplying the total number of eggs in the two grids by 50 (MAFF, 1986).

2.5. Collection of nematode eggs:

Eggs were recovered from faecal samples collected from the animals in the farm, commercial markets and abattoirs of different localities and regions, using the method described by Taylor (1990) and W.A.A.V.P. (Coles *et al.*, 1992).

- i. Homogenize faecal samples with a laboratory stirrer or by placing the faeces in a plastic measuring cylinder with 200 ml of water and plunge up and down with a perforated plunger until all the pellets have been broken up.
- ii. Pour through a 100 mesh (0.15 mm aperture) 20 cm diameter sieve into a bowl. Pour the filtrate into four to eight clayton lane tubes.

- iii. Centrifuge for 2 minutes at about 300 Xg(1500 r.p.m) and gently pour or suck off the supernatant.
- iv. Agitate the tubes to loosen the sediment, then add saturated NaCl solution until a meniscus forms above the tube, add a cover slip and centrifuge for 2 minutes at about 130 Xg (approximately 1000 rev/min on a bench-top centrifuge).
- v. Carefully pluck the cover slip off the tubes and wash off the eggs into a conical glass centrifuge tube, fill with water and centrifuge (2 min about 300 X g).
- vi. Remove the water, re-suspend the eggs in water, estimate the number of eggs per milliliter and dilute to the required concentration.

2.6. Faecal culture for third stage infective larvae:

Individual samples containing 150 eggs of faeces or more were chosen for faecal cultures as recommended by W.A.A.V.P (Coles *etal.*, 1992). The third stage infective larvae were obtained by using the method of Gasmir (2004) and Fadle (1987) by Roberts and O'sullivan (1950). Identification of larvae was achieved following the keys of Georgi (1980) and Soulsby (1986).

Approximately 20 grams of faeces were wrapped in a piece of guaze and then suspended in a close marmalade jar containing a small amount of water to provide the media with moisture. The culture was kept at room temperature for 7 days. On the 8th day the jar was filled with water till it covered the suspended faeces and then left overnight. During

that time the larvae migrate from the wrapped faeces and settled on the bottom of the jar. On the 9th day most of the water in the jar was decanted and a small amount was left. That small amount was distributed into a number of test tubes. The test tubes were left standing for 1 – 2 hours to concentrate the larvae at the bottom. The water on the top was decanted and all the samples were then collected in the one test tube, labeled and stored at 4°C till the time of examination.

From each individual culture, 100 larvae were identified according to the keys used.

2.7. *In-vitro* tests:

2.7.1. The egg hatch test:

The original test was described by LeJamber (1976) and has been used with minor modification by a number of workers (Coles *et al.*, 1992).

Eggs for the egg hatch test must be used within 3 hours of being shed from the host. This can be overcome by anaerobic storage of faecal samples during transit following the method of Hunt and Taylor (1989), as follows:

About 85 ml of tap water was added to 100 ml screw top plastic bottle containing about 8 mm diameter glass beads and about 10 g of freshly collected faeces were added. The lid was screwed on tightly and the bottle shaken vigorously for 1 min to disperse the faecal material, so that the contents of the bottle will rapidly become anaerobic. The bottle was stored at about 20°C and not refrigerated so that eggs for test can be

used up to 7 days after collection. The egg hatch test methodology was ran as follows:

Two ml of fresh eggs collected from the animals survey (less than 3 hours old, or an aerobically stored) (about 150 eggs/ml) were placed in bottles. Then 10 μ l of the anthelmintic solution (ivermetin, abamectin and dorametin or Albendazol drench or levamisole powder) was added to the bottles. The different concentrations of the anthelmintic used were, 0.1, 0.3, 0.5, 0.8, 1, 3, 5, 8, 10, 50 and 100 ng/ml ivermectin, abamectin and doramectin or 0.1, 0.3, 0.6, 0.9, 2, 4, 6, 8 and 10 μ g/ml for levamisole and 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 μ g/ml for Albendazole. Control bottles received solvent only. Then incubated at 27°C for 48 hours, and two drops of lugols iodine were added. At least 100 of the remaining eggs (dead or embryonated) and hatched larvae were counted. The ED₅₀ value was calculated for the eggs by log probit analysis. The test was performed using two replicates per anthelmintic concentration and per control.

2.7.2. Larval paralysis assay:

The procedure was similar to that described by Martin and Le Jambre (1979). The final concentration of levamisole were 0.1, 0.3, 0.6, 0.9, 2, 4, 6, 8 and 10 μ g/ml. 0.1, 0.3, 0.5, 0.8, 1, 3, 5, 8, 10, 50 and 100 ng/ml for abamectin. 1 ml suspension containing 350-416 L₃ was mixed with 1 ml of levamisole and Abamectin solution.

The mixture of L₃ and anthelmintic was incubated in bottles for 24 hour at room temperature. After 24 hours the bottles were read and the larvae were classified as normal (moving) or paralyzed (no observable

motion during 5 seconds). Data were corrected for larval mortality and the LP_{50} values calculated in the concentration of levamisole and Abamectin required to paralyze 50% of the larvae were estimated.

2.8. Statistical Analysis :

The estimation of the ED_{50} and LP_{50} values and log dose curve in anthelmintic resistance were performed using probit analysis by stats direct program ([www.stats direct.com](http://www.statsdirect.com)).

CHAPTER THREE

Results

3.1. Faecal Examination

Examination of 796 faecal samples from sheep originating from different localities in khartuom state revealed presence of different helminth eggs:-

- i. *Strongyle spp.*
- ii. *Strongyloides papillosus.*
- iii. *Trichurs ovis.*
- iv. *Monezia spp.*
- v. *Coccidia spp.*

The highest prevalence of nematode was recorded in october reaching 95.5% *strongyle* egg and 47.1% *Strongyloide* eggs (Table 4). The seasonal prevalence of parasites was showed in (Table 5). And prevalence of pure and mixed infection with gastrointestinal parasites was showed in (Table 6).

For *Coccidia spp* the peak of infection occurred during summer (March- June) 261 (43.5%). While the lowest infection occurred during winter (November –February) 159 (26.5%).

For *Strongyle spp* the highest prevalence was in autumn (July - October) 56 (43.4%) and lowest during summer 31 (24.1%).

For *Strongyloides papillosus.* the peak of infection occurred in autumn 54 (58.0%) and the lowest during winter 17 (18.3%).

For *Monezia* spp. the highest prevalence of infection occurred during winter and summer 22 (42.3%) and the lowest occurred during autumn 8(15.4%).

Trichuris ovis this species was recorded in very low numbers during summer only 2(0.23%).

3.2. Faecal Culture

Faecal cultures composite sample of sheep from different localities revealed presence of third infective stage (L₃) of nematodes belonging to four genera. *Haemonchus contortus* was the predominant nematode species in faecal cultures and *Oesophagostomum* spp had the lowest infection in comparison with other species.

3.2.1. *Haemonchus Contortus*

It is slender larva with a narrow rounded head, ill – defined cells and sheath tail of medium length.

3.2.2. *Trichostrongylus* spp.

Larva has a tapered head, its tail bearing two tuberosities or is indistinctly rounded. It has 16 intestinal cells and the tail of sheath is a short cone.

3.2.3. *Oesophagostomum* spp

Larva has a broad round head, it has 32 intestinal cells and the tail of sheath is filamentous.

3.2.4. *Strongyloides papillosus*

It is small, slender, straight and unsheathed with a long oesophagus and bifid tail.

3.3. Result of *in-vitro* study

3.3.1. Egg hatch assay (EHA)

The results of *in-vitro* egg hatch assay were showed in (Table 7). The ED₅₀ value was evaluated and resistance was confirmed in ivermectin (482.444521 ng/ml) (Figure 1), Dromectin (256.525577 ng/ml) (Figure 3) and levamisole (6.924595 µg/ml) (Figure 4). On the other hand, Abamectin showed no resistance (7.410285 ng/ml) (Figure 2) and Albendazole showed susceptible status (0.003162 µg/ml) (figure 5).

3.3.2. Larval paralysis assay (LPA)

The results of *in-vitro* larval paralysis assay (LPA) were performed using Levamesole and Abamectin (Table 8). Resistance was not detected in levamisole the LP₅₀ value was (0.890862 µg/ml) (Figure 6). On the other hand, Abamectin showed susceptible status (0.000107 ng/ml) (Figure 7).

Table 4: prevalence of different gastrointestinal parasites during the periode June 2005-May 2006 No. (%):-

Parasite Species	Jun 2005	Jul 2005	Aug 2005	Sept 2005	Oct 2005	Nove 2005	Dece 2005	Janu 2006	Feb 2006	Mar 2006	Apr 2006	May 2006	Total (%)
<i>Coccidia spp</i>	63 (84.0)	81(75.7)	24 (96.0)	60 (88.2)	15 (88.2)	17 (70.8)	32 (88.9)	26 (76.5)	84 (75.0)	22 (48.9)	76 (86.4)	100(60.6)	75.4
<i>Strongyle spp</i>	11 (14.7)	14 (13.1)	4 (16.6)	25 (36.8)	13 (96.5)	5 (20.8)	8 (22.2)	10 (29.4)	19 (17.0)	3 (6.7)	2 (2.3)	15 (9.1)	16.2
<i>Strongyloides Papillosus</i>	8 (10.7)	19 (17.8)	4 (16.6)	23 (33.8)	8 (47.1)	11 (45.8)	2 (5.6)	-	4 (3.6)	1 (2.2)	3 (3.4)	10 (6.1)	11.7
<i>Monesia spp</i>	2 (2.7)	2 (1.9)	1 (4.0)	5 (7.4)	-	-	9 (25.0)	4 (11.8)	9 (8.0)	7 (15.6)	5 (5.7)	8 (4.8)	6.5
<i>Trichuris ovis</i>	2 (2.7)	-	-	-	-	-	-	-	-	-	-	-	0.3
Total No. of samples	75	107	25	68	17	24	36	34	112	75	88	165	796

Table 5: Seasonal prevalence of different gastrointestinal parasites during the period June 2005-May 2006 No. (%):-

Parasite Species	Autumn	Winter	Summer	Total
<i>Coccidia spp</i>	180 (30.0)	159 (26.5)	261 (43.5)	600 (68.5)
<i>Strongyle spp</i>	56 (43.4)	42 (32.5)	31 (24.1)	129 (14.7)
<i>Strongyloides Papillosus</i>	54 (58.0)	17 (18.3)	22 (23.7)	93(10.6)
<i>Monesia spp</i>	8 (15.4)	22 (42.3)	22 (42.3)	52 (5.9)
<i>Trichuris ovis</i>	0	0	2 (100)	2 (0.23)
Total No. of Samples	296 (34.0)	240 (27.4)	338 (38.8)	876

Autumn (July ——— October)

Winter (November ——— February)

Summer (March ——— June)

Table 6 : prevalence of pure and mixed infections with gastrointestinal parasites during the June 2005 - May 2006

Sample	Prevalence (%)
Samples having one parasites	461 (57.9)
Samples having two parasites	150 (18.9)
Samples having three parasites	52 (6.5)
Samples having four parasites	5 (0.6)
The negative samples	128 (16.1)

Table 7: Result of *in-vitro* studies.
Egg hatch assay (EHA)

Test	Drug used	ED ₅₀
Egg hatch assay	Albendazole	0.003162µg/ml
Egg hatch assay	Ivermectin	482.444521ng/ml
Egg hatch assay	Abamectin	7.410285 ng/ml
Egg hatch assay	Doramectin	256.525577ng/ml
Egg hatch assay	Levamisole	6.924595 µg/ml

The results showed resistance for ivermectin, doramectin and levamisole, but when used albendazole and abamectin the results showed no resistance.

Table 8: Result of *in-vitro* studies.
Larval paralysis assay(LPA)

Test	Drug used	LP ₅₀
Larval paralysis assay	Levamisole	0.890862µg/ml
Larval paralysis assay	Abamectin	0.000107ng/ml

The results showed no resistance for levamisole and abamectin.

Figure 1: Ivermectin Dose Response Curve (EHA)

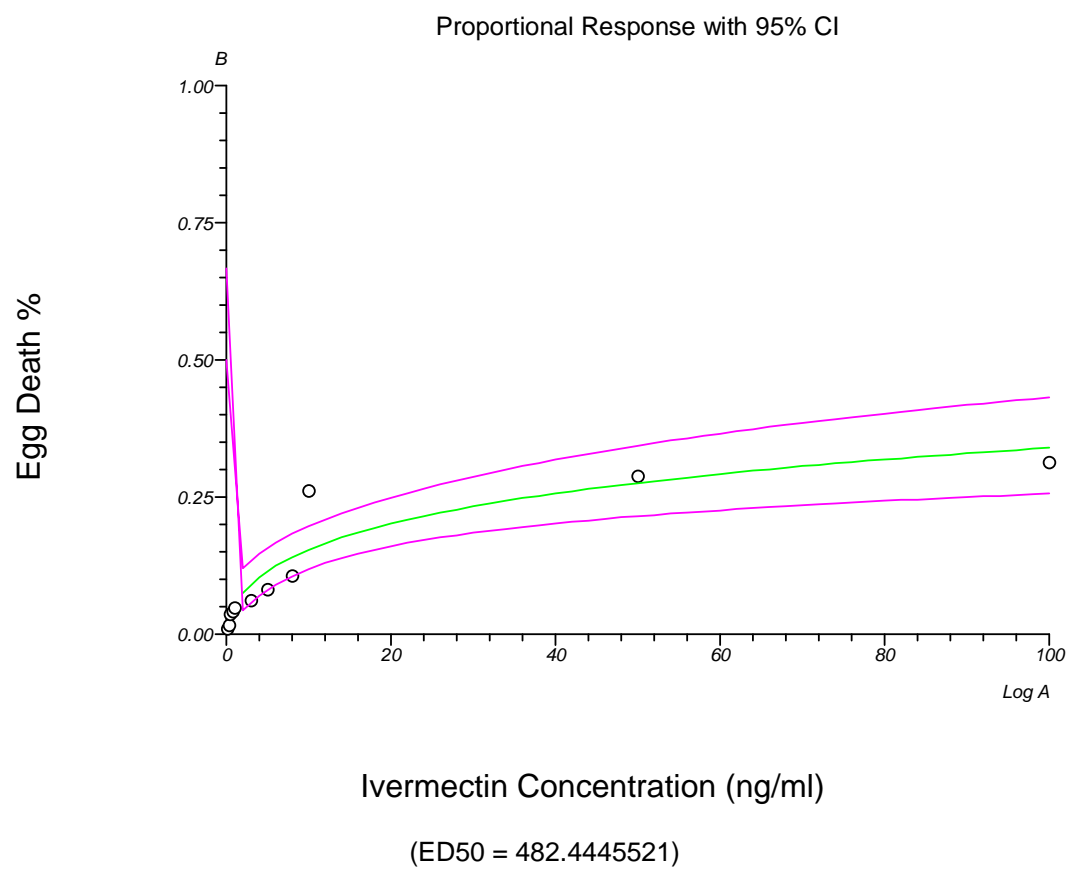


Figure 2: Abamectin Dose Response Curve (EHA)

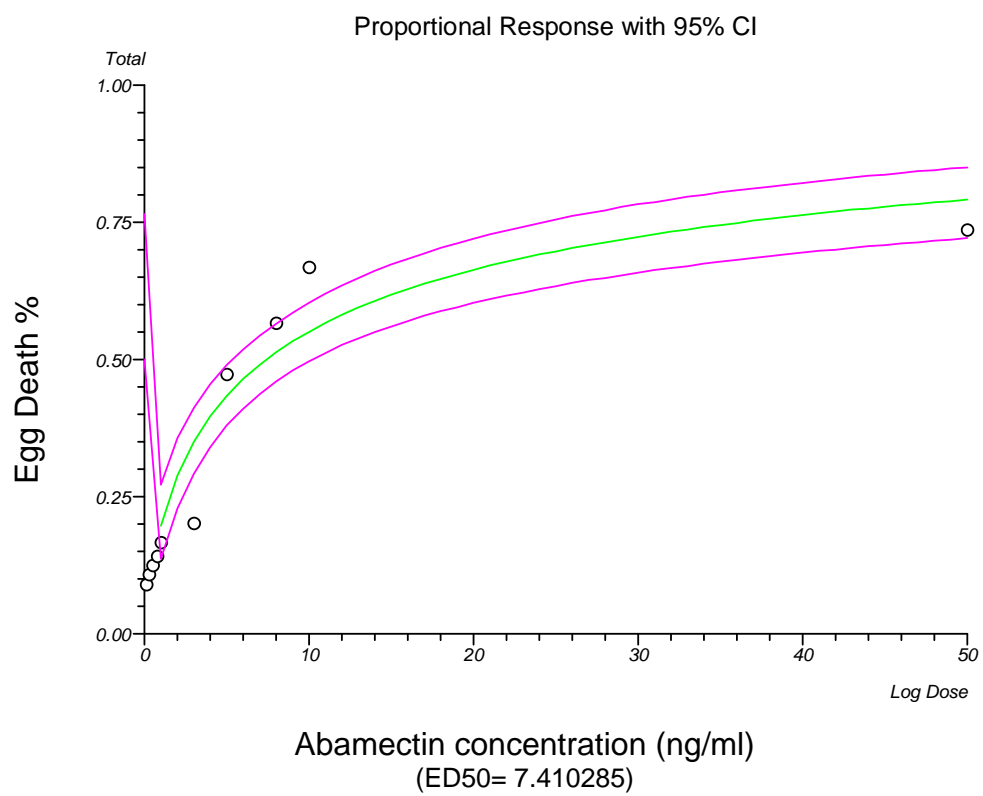


Figure 3: Doramectin Dose Response Curve (EHA)

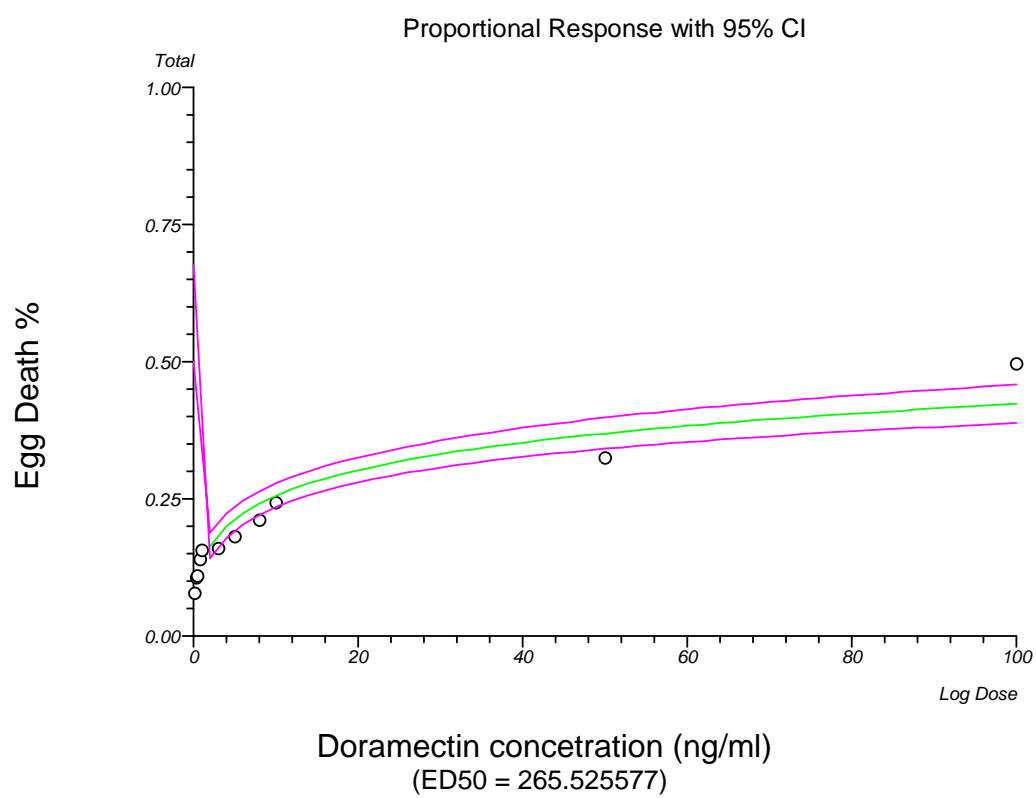


Figure 4: Levamisole Dose Response Curve (EHA)

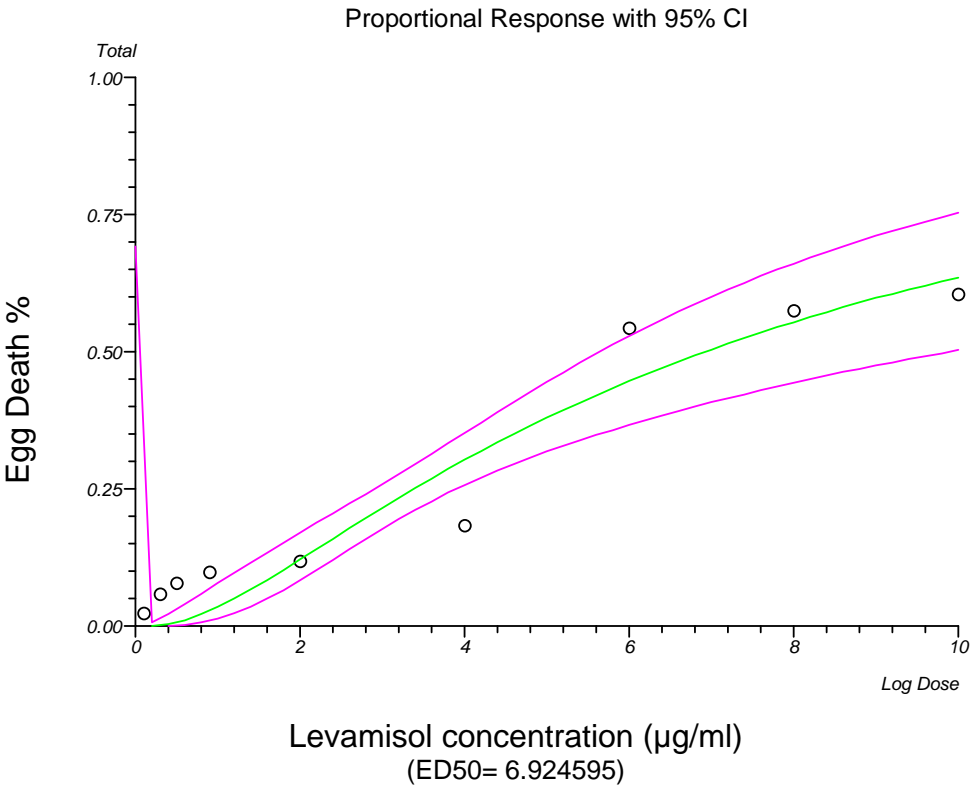


Figure 5: Albendazole Dose Response Curve (EHA)

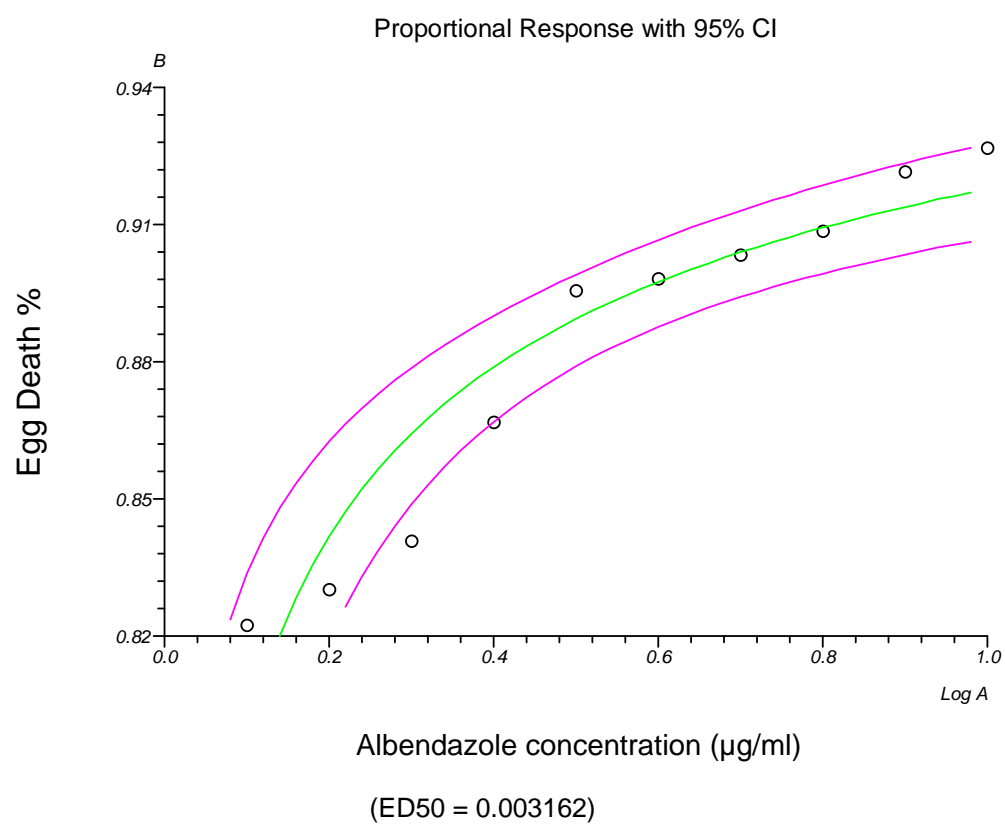


Figure 6: Levamisol Dose Response Curve (LPA)

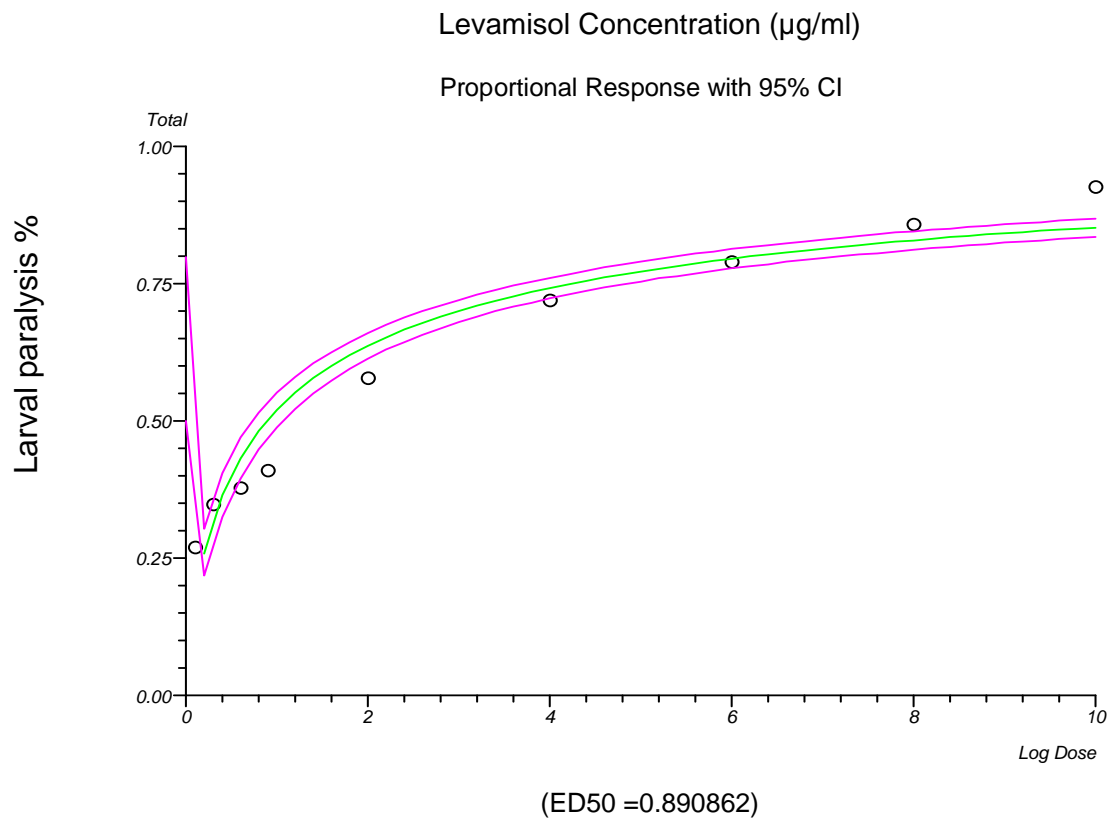
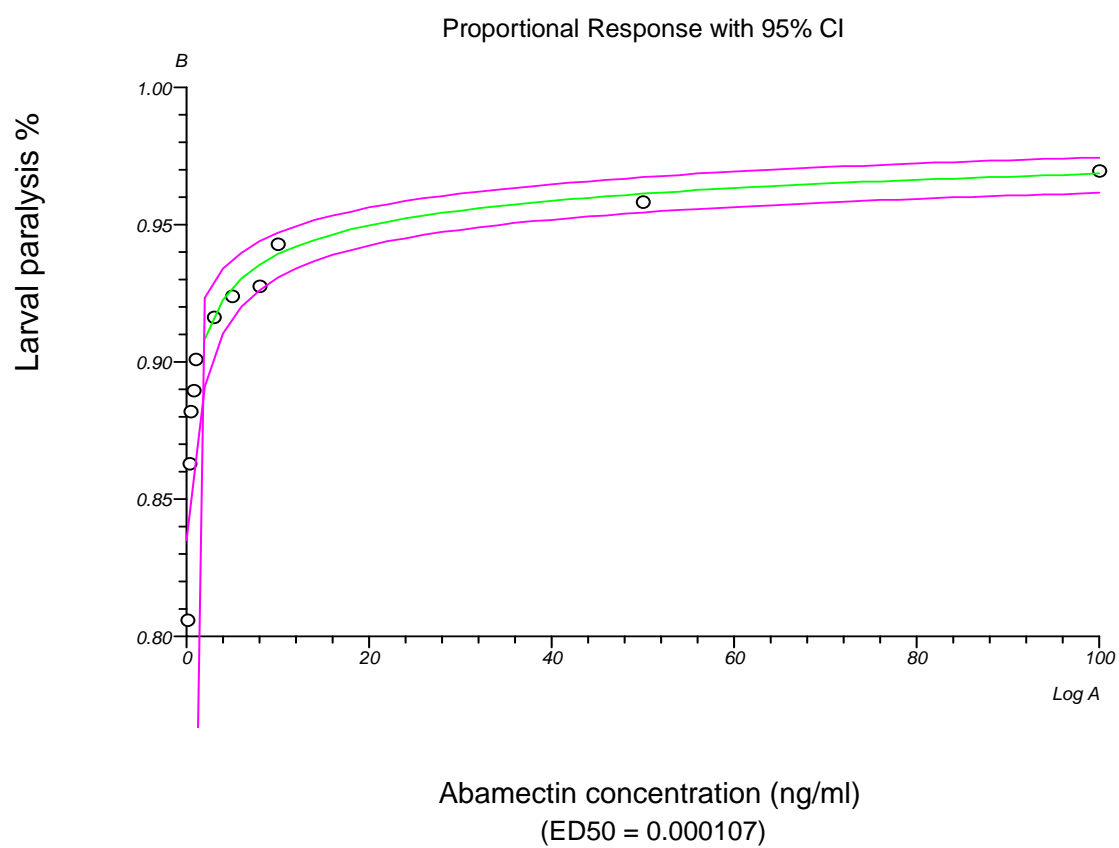


Figure 7: Abamectin Dose Response Curve (LPA)



CHAPTER FOUR

Discussion

In the present study a survey of gastrointestinal nematodes of sheep was carried at Khartoum state during the period June 2005 – May 2006. 796 faecal samples were collected. After faecal culture the study revealed the presence of third infective larval (L₃) of nematodes belonging to four genera. The species identified were, *Haemonchus contortus*, *Trichostrongylus* spp. *Oesophagostomum columbianum* and *Strongyloides papillosus*.

Several authors reported the same nematodes infections in sheep in Sudan (Gagoad and Eisa, 1968; Eisa and Ibrahim, 1970; ELBadawi *et al.*, 1978; Atta EL Mannan, 1983; Ahmed and ELMalik, 1997; Ghada, 2000; and Gasmir (2004). Most of these last authors reported parasite species belonging to four different genera namely *Haemonchus contortus*, *Trichostrongylus* spp, *Oesophagostomum* spp and *Strongyloides papillosus*. Further more they stated that *Trichostrongylus* spp had the lowest frequency of infection in comparison with other species. These difference in results be due to the fact that they used both faecal examination and post-mortem procedure for determination of parasites in sheep whereas Ahmed and Elmalik used only faecal examination techniques.

In this study Protozoa class *Coccidia* were found with higher prevalence in all the seasons, this result agree with Abaker (1996). In addition to this, in this study present Cestodes were found to be less common than nematodes. The genus identified was *Monezia* with high prevalence in both Winter and Summer. Ghada (2000) reported high

prevalence of *Moniezia expansa* during winter. This might be attributed to availability of oribatid mites in pasture. In Argentina, Denegri and Alzued (1992) reported seasonal variation of oribatid mites and increase in its number coincided with increase in mean temperature and rainfall. Information about the seasonal occurrence of oribatid mites is not available in the Sudan. It may be inferred that oribatid mites occurred in high numbers in pasture during the rainy season. The mites ingest *Moniezia* egg and *onchospheres* take approximately four months to reach infective stage Soulsby (1986). Sheep ingest infected mites and the prepatent period is 37-40 days Soulsby (1986). So the increase in frequency of *Moniezia* spp. Egg would be expected to occur during winter.

In the present study *in-vitro* assays for detection of anthelmintic resistance were made (Table 6 and 7). Taylor (1990) defined specific cut-off mean inhibitory concentration (MIC) values for thiabendazole (0.1 µg/ml), Levamisole (1.0 µg/ml) and Ivermectin (8.0 ng/ml).

The first indication of the ivermectin resistant by *Haemonchus contortus* in sheep occurred in 1986 in South Africa. Carmichael *et al.* (1987); Vanwyk and Malan (1988) and Vanwyk *et al.* (1989) reported isolates and probable existence of five additional ivermectin resistant strain of *Haemonchus contortus*. Wooster *et al.* (2001) reported ivermectin and closantel resistance against *Haemonchus contortus* in sheep.

The egg hatch assay revealed the ED₅₀ of 482.444521 ng/ml for ivermectin and 6.924595 µg/ml for levamisole, this study indicated

resistance of nematodes, to ivermectin and levamisole and this agree with the results of Sivaraj *et al.* (1994) and Dharendra- singh *et al.* (1995) who reported 30.48 µg/ml for levamisole.

Toylor and Hunt (1989) reported in England and Wales two species of ruminant nematodes *Haemonchus contortus* and *Ostertagia circumcincta*, have been confirmed to developed resistance to benzimidazole anthelmintics. also in Australia (waller, 1986) and in New Zealand (Mckenna, 1989) many species are now resistant. *Cooperia curticei* has become resistant to benzimidazoles in Netherland (Borgsteede, 1986). The results disagreed with these studies but Bersissa and Abebe (2006) reported the ED₅₀ in Albendazole drug against *Heamonchus contortus* 0.06 µg/ml, this result agree with the study which present the ED₅₀ of Abendazole in egg hatch assay 0.0003162 µg/ml. The result of egg hatch assay in comparison with know that nematodes with highly susceptibility to Albendazole.

The level of levamisole resistance as measured in larval paralysis assay by Gasmir (2004) who found 1.3 µg/ml and Varady *et al.* (1995) reported greatly increased ED₅₀ values during the patency period of *Heamonchus contortus* resistance isolate. This result disagree with study which present no resistance in levamisole 0.890862 µg/ml.

The level of levamisole resistance asmeasured by egg hatch paralysis assay is always high and variable (Sangster, *et al.* 1988; Varady, *et al.* 1999). The between – assay variation of different resistant isolates is very clear. Varady *et al.* (1999) obtained ED₅₀ of 12µg/ml levamisole in the frist assay, which was carried out at the beginning of the patent period, up to 994µg/ml levamisole in the last assay performed

two weeks later. Similarly Varady *et al.* (1995) reported greatly increased ED₅₀ values during the patency period of *Haemonchus contortus* resistant isolate. the exact reason for the rise in ED₅₀ values is unknown.

The level of abamectin when measured by egg hatch assay and larval paralysis assay give same result. The LP₅₀ in larval paralysis assay was 0.000107 ng/ml and this means abamectin is not resistance. and in egg hatch assay the ED₅₀ was 7.410285 ng/ml and this means abamectin allso is not resistant. Kaplan, *et al.* (1994) reported Abamectin was judge to be safe and effective, this result agrees with the results of this study. Like wise, the result showed that the ED₅₀ for doramectin when used in egg hatch assay was 265.525577ng/ml this means doramectin is resistance. Nevertheless Diez-Baños, *et al.* (2008) analyzed the presence of resistance to benzimidazole and macrocyclic lactones performed in sheep under field condition using *in-vivo* Faecal Egg Count Reduction Test (FECRT) and *in-vitro* Egg hatch assay (EHA) and the result showed simultaneously resistance in both test.

Conclusion and Recommendations

The first study for anthelmintic resistance in Sudan since 1990 , however, the situation of anthelmintic resistance until now is unclear, because the Sudan is a large country and various climatic zone with high different animal. This needs different studies of anthelmintic resistance (*in vivo* or *in vitro*) in all states of the Sudan . The all accumulation of these studies will give a complete and full picture of anthelmintic resistance in the Sudan.

Conclusion

- i. In conclusion this study is considered an addition to the scanty information available on the natural infection of Sudanese sheep by gastrointestinal nematodes.
- ii. According to this study *Coccidia Spp.* is prevalent in all periods of survey.
- iii. *Haemonchus contortus* is the most common nematode parasite in the Sudanese sheep, and *Trichostrongylus axei* is more or less common in sheep.
- iv. Abamectin in two testes (EHA and LPA) presence no resistance but Levamisole presents resistance in the (EHA) and not resistance in (LPA).
- v. Doramectin and ivermectin presents resistance in (EHA).
- vi. Albendazole gave a good result in (EHA) and presence no resistance.

Recommendations

The use of *in-vitro* methods . for field screening of anthelmintic resistance of sheep nematodes is recommended as it is more economic and suitable under Sudan conditions.

Further *in-vivo* and *in-vitro* studies were recommended to study levamisole resistances in sheep nematode.

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